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# A catch-and-release approach to selective modification of accessible tyrosine residues

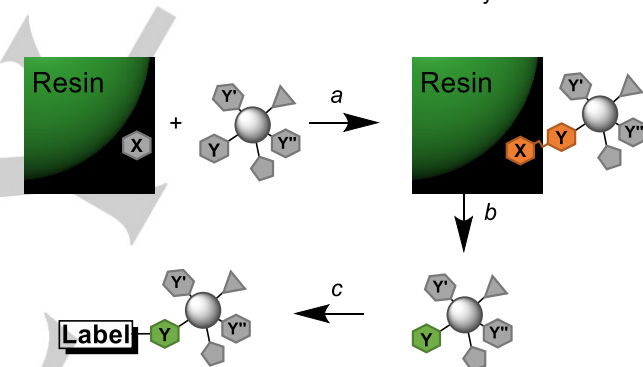
Christopher Allan,<sup>[a]</sup> Miroslav Kosar,<sup>[a]</sup> Christina V. Burr,<sup>[a]</sup> C. Logan Mackay,<sup>[a]</sup> Rory R. Duncan,<sup>[b]</sup> and Alison N. Hulme<sup>\*[a]</sup>

**Abstract:** The tyrosine side chain is amphiphilic leading to significant variations in the surface exposure of tyrosine residues in the folded structure of a native sequence protein. This variability can be exploited to give residue-selective functionalization of a protein substrate by using a highly reactive diazonium group tethered to an agarose-based resin. This novel catch-and-release approach to protein modification has been demonstrated for proteins with accessible tyrosine residues, which are compared with a control group of proteins in which there are no accessible tyrosine residues. MS analysis of the modified proteins showed that functionalization was highly selective, but reactivity was further attenuated by the electrostatic environment of any individual residue. Automated screening of PDB structures allows identification of potential candidates for selective modification by comparison with the accessibility of the tyrosine residue in a benchmark peptide (GYG).

Selective protein modification is an important tool in Chemical Biology, allowing the attachment of a synthetic label to a functional biomolecule in a controlled manner. The resulting bioconjugate may be used in fluorescence imaging,<sup>[1,2]</sup> in the study of post-translational modifications (PTMs)<sup>[3-6]</sup> or in the development of novel protein therapeutics.<sup>[7-10]</sup> Modern approaches to bioconjugation include the incorporation and subsequent modification of unnatural amino acids, or modification of a native amino acid side chain, most often lysine or cysteine.<sup>[11-13]</sup> As an alternative native target, tyrosine (Tyr) is an attractive prospect due to its low frequency of occurrence and varied surface exposure, which allows discrimination between multiple residues of the same type. To this end, we report the development of a novel catch-and-release protein-tagging strategy, which targets a Tyr residue in a native sequence protein (Figure 1). By using a chemoselective electrophile which is tethered to a resin bead, protein modification is achieved through a surface-surface interaction between the protein and the resin. Compared to a small molecule electrophile in solution, this solid-phase approach provides enhanced selectivity for highly exposed residues.

The Tyr side chain is amphiphilic,<sup>[14]</sup> which leads to significant variation in the surface exposure of Tyr residues in the folded structure of any protein (SI Section 2). A number of modification strategies have been reported which selectively

target Tyr residues, these include the use of the diazodicarboxamide reagent PTAD,<sup>[15]</sup> a three-component Mannich-type reaction,<sup>[16]</sup> and several metal-mediated transformations.<sup>[17-19]</sup> The preferred modifying reagent for the current work is a diazonium salt as it forms an azobenzene moiety upon reaction with Tyr in polypeptides/proteins,<sup>[20-22]</sup> and has shown good selectivity for reaction with Tyr over competitive reaction sites such as His, Lys and Cys at near-neutral pH;<sup>[22]</sup> this constitutes the 'catch' step of the protein modification strategy (Figure 1). The azobenzene group can then be cleaved under mild conditions using dithionite (SI Section 3), allowing the release of the protein from the solid-phase platform.<sup>[23-26]</sup> The cleavage product bears an *o*-aminophenol modification which may be used as a functional handle for the attachment of a synthetic label.



**Figure 1.** A catch-and-release approach to protein modification. (a) Catch process: the protein is immobilized onto the support matrix through covalent attachment of a reactive residue (Y) to a reactive group (X). Selectivity is achieved through a combination of steric and/or electronic factors. (b) Release process: mild conditions are used to cleave the labile linker between the protein and the solid-phase. The released protein carries a modification or tag arising from the anchor point to the resin. (c) The protein tag acts as a functional handle to which a synthetic label can be attached.

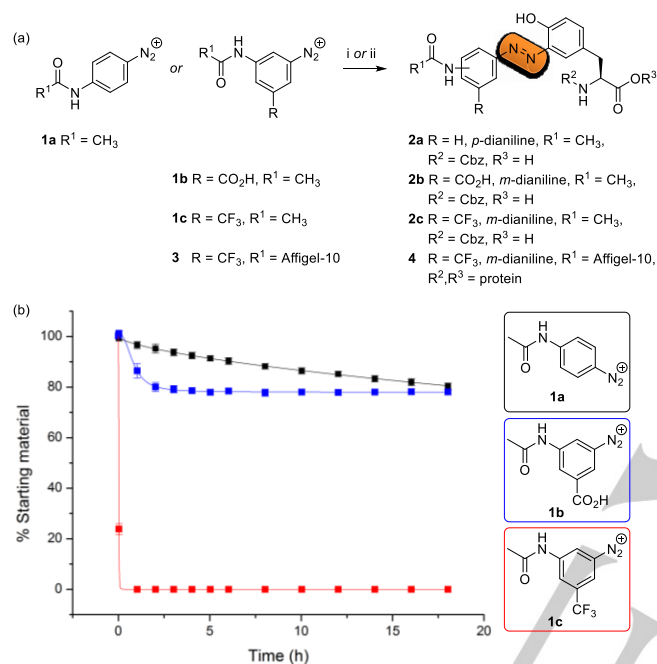
The solid-phase nature of the proposed catch-and-release platform allows step-wise separation of the diazotization reaction which forms the reactive electrophile, and the introduction of the protein substrate. This spares the protein from the potentially denaturing acidic conditions required for diazotization, whilst also allowing the recovery of unbound protein in neutral pH buffer with no other additives. However, our initial studies indicated that incubation of proteins with resin-bound electrophiles over extended periods (>24 h), resulted in multiple labelling events and lowered overall protein recovery; even under the "protein-friendly" labelling conditions enabled by the on-resin approach. It has been reported that electron-poor diazonium salts are more highly reactive to nucleophilic attack,<sup>[20,27-30]</sup> while efficient reductive cleavage of an azobenzene is achieved when the diazo group is situated between an electron-rich and an electron-poor arene.<sup>[31]</sup> With this in mind, three diazonium salts which could be linked to

[a] C. Allan, M. Kosar, C. V. Burr, Dr. C. L. Mackay, Prof. A. N. Hulme  
EaStCHEM School of Chemistry, University of Edinburgh, Joseph  
Black Building, David Brewster Road, Edinburgh, EH9 3FJ (UK)  
E-mail: Alison.Hulme@ed.ac.uk

[b] Prof. R. R. Duncan  
Institute of Biological Chemistry, Biophysics and Bioengineering,  
David Brewster Building, Heriot-Watt University, Edinburgh, EH14  
4AS (UK)

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the NHS-functionalized agarose-based resin Affi-Gel 10,<sup>[32]</sup> were screened for their comparative reactivity in a coupling reaction with the model residue Cbz-Tyr-OH (**1a-c**, Figure 2, SI Section 4). This screening indicated that the diazonium salt derived from a *meta*-substituted dianiline bearing a CF<sub>3</sub> group (**1c**) might give the desired reactivity as the capturing electrophile. Thus this linker design was adopted for application on-resin using Affigel-10. The immobilized aniline was functionalized as its diazonium salt **3**, to capture surface accessible tyrosine residues in proteins (as in **4**, Figure 2a).



**Figure 2.** (a) Coupling reaction between diazonium salt models for the resin-linked electrophile and Cbz-Tyr-OH. Reagents and Conditions: (i) Cbz-Tyr-OH, phosphate buffer (pH 7.2), rt, time intervals as shown in (b); or (ii) protein, phosphate buffer (pH 7.2), rt, 24 h. (b) Relative reaction rates for the coupling of diazonium salts **1a-c** with Cbz-Tyr-OH as monitored by UHPLC; reactions performed in quadruplicate and presented as the mean  $\pm$  S.E.M.

A group of six proteins (RNase A, SBTI,  $\beta$ -Lac, Mb, HEWL and Cyt C) was chosen to test the new catch-and-release platform, each of which contained at least one tyrosine residue. The Tyr surface exposure in each protein was predicted using the solvent-accessible surface area (SASA) calculation in PyMOL (SI Section 5).<sup>[33]</sup> This allowed these proteins to be separated into two categories: those with solvent accessible Tyr residue(s) where there was (at least) one Tyr residue with a SASA value greater than, or equivalent to, the SASA value of a benchmark tripeptide GYG (23.10 Å<sup>2</sup>);<sup>[34]</sup> and those where there were no solvent accessible Tyr residues as determined using the same benchmark. The catch-and-release protocol was performed on each of these proteins using optimized conditions: (i) incubation of the protein with the highly activated resin-bound diazonium electrophile **3**,<sup>[35]</sup> followed by washing and removal of any unbound protein; then (ii) cleavage of the captured protein **4** under mild dithionite conditions, resulting in regeneration of the resin-

bound aniline and release of the selectively modified protein (Table 1). The accessibility of Tyr residues in the native protein was reflected in the yield of modified protein obtained; the poor yields of modified protein for Mb, Cyt C and  $\beta$ -Lac strongly suggesting that an accessible Tyr residue is a requirement for the capture process to work. Reassuringly, in all cases the total protein recovery was found to be >96% and for the three proteins with accessible Tyr residues it was calculated that the conversion based on recovered protein ranged from 71-93%.

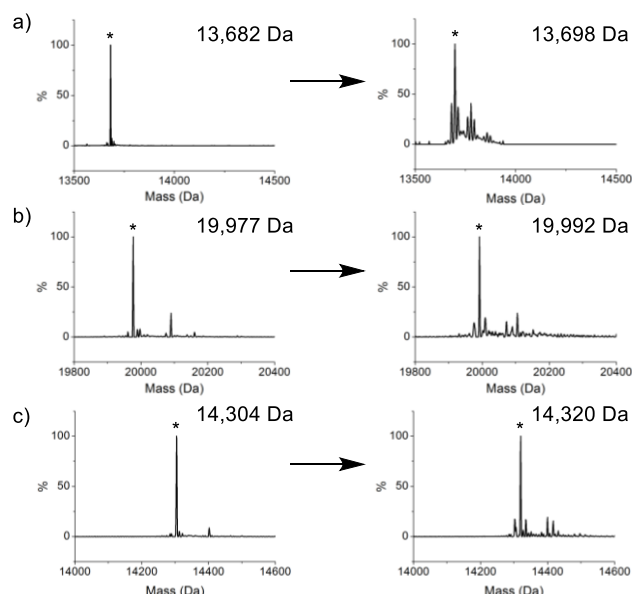
**Table 1.** Yield of modified protein and recovered protein in the catch-and-release protocol.<sup>[a],[b]</sup>

Protein	Modified Protein (%)	Unmodified Protein (%)	Total Protein Recovery (%)	Highest Tyr SASA (Å <sup>2</sup> ) <sup>[c]</sup>
RNase A	32.8	64.7	97.5	27.6 (Y076)
SBTI	13.5	83.5	97.0	37.9 (Y062)
HEWL	13.8	80.5	94.3	21.7 (Y020)
Mb	2.6	95.5	98.1	8.2
Cyt C	1.8	94.6	96.4	11.2
$\beta$ -Lac	0.6	95.5	96.1	15.3

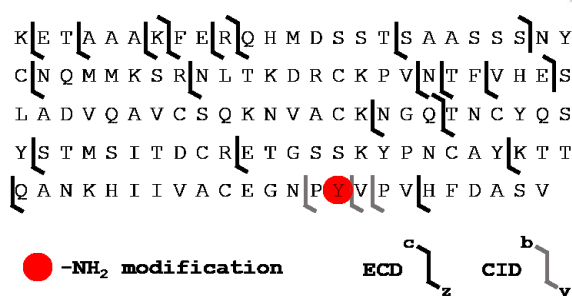
<sup>[a]</sup> Details of the optimized protocol provided in the Experimental Section.

<sup>[b]</sup> Dashed line represents the separation between proteins with accessible Tyr residues and those without. <sup>[c]</sup> PyMOL SASA calculation performed on PDB files: 4J5Z; 1BA7; 4YM8; 5D5R; 2B4Z; and 3NPO respectively.

To assess the selectivity of the optimized protocol, the three proteins with the highest catch-and-release conversions (RNase A, SBTI and HEWL) were analyzed by MS. In each case the singly modified protein was the predominant species, and only minor amounts of unmodified and twice modified protein were also observed (Figure 3, SI Section 6). Bottom-up analysis allowed the modification sites to be identified as: Y115 in RNase A; Y062 in SBTI; and either Y020 or Y023 in HEWL (SI Section 7). This bottom-up analysis also showed no evidence of any histidine cross-reactivity in each of the three proteins studied. RNase A provides a particularly challenging target for this methodology as it has three accessible tyrosine residues (Y076, Y092 and Y115). Hence further evidence was sought to confirm the site of modification in this protein. Top-down fragmentation was performed on the isolated singly modified species by electron-capture dissociation (ECD, SI Section 8) and collision-induced dissociation (CID, SI Section 9). Combining the results from each method allowed unequivocal separation of the modified site from other potentially reactive residues, and the site of the +15 Da modification was confirmed as Y115 (Figure 4). By comparison, MS analysis of RNase A modified in solution (using the small nitrating agent tetranitromethane (TNM),<sup>[36,37]</sup> and subsequent reduction) was shown to give the 4x modified protein as the major product (SI Section 10);<sup>[38]</sup> further confirming that the observed Tyr residue selectivity could be attributed to the catch-and-release approach.



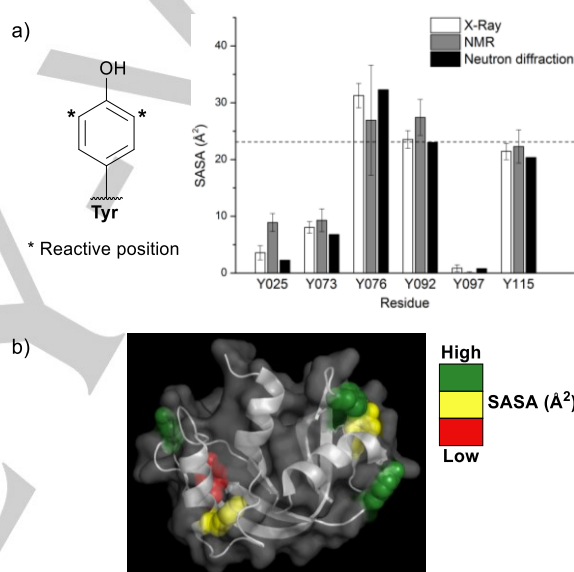
**Figure 3.** MaxEnt deconvoluted spectra following LC-MS analysis of: (a) RNase A (theoretical mass unmodified protein: 13,682 Da); (b) SBTI (theoretical mass unmodified protein: 19,978 Da); and (c) HEWL (theoretical mass unmodified protein: 14,305 Da), showing parent protein (left) and predominantly singly modified protein (+15 Da, right). Proteins were incubated with the modified Affigel-10 resin **3** for 24 h, washed to elute unbound protein from **4** and released by incubation with dithionite for 18 h.



**Figure 4.** Top-down fragmentation of catch-and-release modified RNase A by ECD and CID. The peptide fragment ions identified by ECD (SI Section 8) and CID (SI Section 9) experiments are overlaid on the RNase A amino acid sequence, confirming the site of modification as residue Y115 (identified in red).

To better understand the selectivity of the catch-and-release method, the reactive residues in RNase A were examined using deposited structures in the Protein Data Bank.<sup>[39]</sup> 75 X-ray structures of native sequence RNase A were retrieved, as well as structures from NMR and neutron diffraction studies (Figure 5; SI file). In order to expedite the SASA calculation of reactive Tyr sites in each of these structures, a SASA automation script was developed (SI file) which allows the rapid ranking of the reactive residues according to their exposure. The Tyr solvent-accessibility profile for RNase A was well conserved among X-ray, NMR and neutron diffraction derived structures. Since top-down fragmentation provides no evidence to support labelling of the other exposed residues (Y076 & Y092), this suggests that high

surface exposure is important for labelling in the catch-and-release system, but there may be other contributing factors which help determine selectivity. The three most exposed Tyr residues were next examined for intramolecular H-bonding, which might explain the difference in reactivity (SI Section 11). However, none of the exposed residues were found to interact with any functional groups which would dramatically alter their nucleophilic potential and so no distinction could be made between them. Finally, through plotting the protein contact potential, it was observed that both Y076 and Y092 are found in close proximity to positively charged residues, while Y115 is found on a relatively uncharged protein surface (SI Section 12). With the resin-bound diazonium salt holding a positive charge, the effects of electrostatic repulsion may therefore help explain the selectivity observed between these three residues.



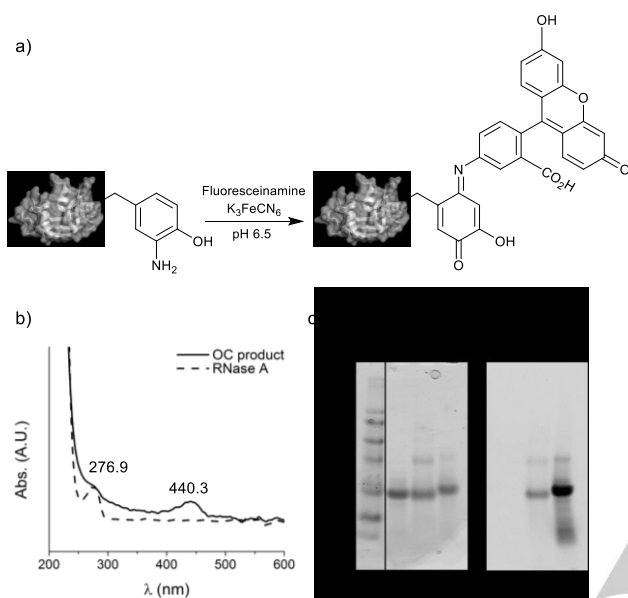
**Figure 5.** SASA results for Tyr residues in RNase A. (a) SASA results for the reactive positions of the Tyr phenol ring using X-ray ( $n=75$  deposits), NMR ( $n=32$  conformers) and neutron diffraction derived structures. The dashed line represents the SASA output of a modelled tripeptide, GYG, which was used as a benchmark of high exposure (SASA: 23.10 Å<sup>2</sup>). (b) The structure of RNase A with Tyr residues ranked by SASA results. (PDB ID: 4J5Z).

To demonstrate the utility of the *o*-aminophenol modification, a fluorophore was conjugated to the catch-and-release modified RNase A using an oxidative coupling strategy developed by Francis *et al.*<sup>[37,40]</sup> (Figure 6). Using a fluorescent aniline (fluoresceinamine) and a biocompatible oxidizing agent (K<sub>3</sub>Fe(CN)<sub>6</sub>), the catch-and-release modified protein was fluorescently labelled at room temperature and near neutral pH (Figure 6a). The reaction was monitored by HPLC-PDA, and incorporation of the fluorophore was identified by a new absorbance peak at 440.3 nm (Figure 6b). The fluorescently labelled protein was also compared to unmodified and FITC-labelled RNase A standards by SDS-PAGE (Figure 6c). With ten Lys residues in the target protein and an excess of the FITC reagent, the fluorescent band resulting from the FITC-labelled standard represents multiple, non-selective labelling and the



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fluorescently labelled catch-and-release product compares favorably to this. While this example shows incorporation of a fluorophore, the post-cleavage functionalization step may be used to incorporate a versatile range of functional molecules, providing they can be equipped with a nucleophilic aniline. In future, this may be expanded to other fluorescent labels, polyethylene glycol polymers or cytotoxic compounds.



**Figure 6.** Oxidative coupling of an aniline with catch-and-release modified RNase A. (a) The oxidative coupling conditions used for the reaction. (b) Conjugation of the fluorophore resulted in a new absorbance at 440.3 nm. (c) SDS-PAGE analysis of the oxidative coupling product. Unmodified RNase A and a FITC-labelled RNase A sample were compared with the oxidative coupling product using Coomassie stain and fluorescence scanning.

A novel system has been developed to allow the selective modification of native sequence proteins using a solid-phase platform. Catch-and-release modification provides singly-modified protein at a defined location while at the same time allowing the efficient recovery of any unmodified protein. The selectivity of this method relies primarily upon steric control, thus proteins which have tyrosine residues of only low solvent accessibility and SASA scores less than that of the benchmark tripeptide GYG (exemplified in this study by  $\beta$ -Lac, Mb and Cyt C) are not likely to be captured and modified in high yield. However, for protein candidates with accessible tyrosine residues (exemplified in this study by RNase A, SBTI and HEWL), reactivity might be further attenuated by factors such as electrostatic repulsion. In addition to providing a selective labelling strategy for native sequence proteins, we believe that the immobilization chemistry described may find future use in profiling the accessibility of Tyr residues in proteins with undefined structure and in biophysical applications such as SPR or SERS, where control over the anchor point of a protein to a solid-phase would ensure homogeneous presentation of the immobilized biomolecule.

## Experimental Section

**Catch-and-release protocol:** 5-(Trifluoromethyl)-1,3-phenylenediamine (27 mg, 150  $\mu$ mol) was dissolved in 3 mL DMSO/IPA (10:90) and added to Affi-Gel 10 resin (1 mL, 15  $\mu$ mol/mL loading of NHS-ester) in a 5 mL SPE tube with a polyethylene frit. The resin mixture was agitated on a rotator for 4 h at rt and then washed with 5 column volumes (CV) of MeOH. Ethanolamine (10  $\mu$ L, 166  $\mu$ mol) was added to the resin with 3 mL IPA, and the mixture was agitated for 1 h at rt. The resin was drained and washed with 3 CV IPA and 3 CV phosphate buffer (100 mM, pH 7.2).  $NaNO_2$  (35 mg, 507  $\mu$ mol) was dissolved in acetic acid solution (3 mL, 600 mM, pH 2.5) and added to the resin which was agitated on a rotator for 1 h at rt. The resin was washed with 2 CV buffer and protein (3 mL, 1.95 mM in buffer) was added. The reaction mixture was agitated on a rotator for 24 h at rt. The resin was washed with 25 CV buffer and the eluate was analysed by UHPLC. Once the eluate was clear of protein, aqueous  $Na_2S_2O_4$  (3 mL, 300 mM) was added and the resin was agitated on a rotator for 18 h at rt. The cleaved, modified protein was collected by draining the resin, which was then washed with 1 CV phosphate buffer (100 mM, pH 7.2). The sample volume was reduced to  $\sim$ 50  $\mu$ L using a MWCO spin cartridge (Amicon Ultra, 3,000 NMWL) and desalted by washing with phosphate buffer (5 x 400  $\mu$ L, 100 mM, pH 7.2).

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**Keywords:** catch-and-release • diazonium • amino-tyrosine • solvent-accessible surface area • native sequence

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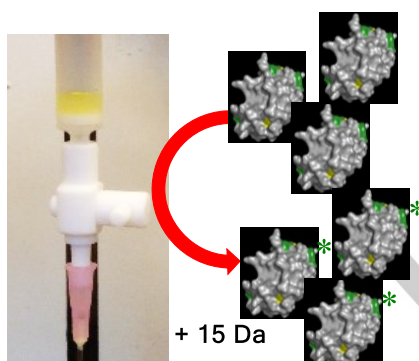
## Entry for the Table of Contents

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**Selectivity through accessibility:**

On-resin capture of solvent-accessible tyrosine residues by a highly reactive resin-bound electrophile allows selective modification of native sequence proteins.



*C. Allan, M. Kosar, C. V. Burr, C. L. Mackay, R. R. Duncan, A. N. Hulme\**

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